Microbial networks for bioremediation of chlorinated ethenes

Vargas Lopez, R.

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Chapter 6

General discussion

The design of a Perchloroethylene-remediating network

This thesis reports on the design, realization, performance and optimization of a new network aimed at the microbial remediation of environmental pollution by perchloroethylene (PCE). The network consists of three living components and an external carbon and energy source (Fig. 1A). It should in principle be capable of removing PCE robustly. The living components are the specific microorganism *Desulfitobacterium hafniense* Y51 and two microbial consortia. One consortium should provide electrons (redox equivalents) in a form that *D. hafniense* Y51 can use to reduce PCE. For the system to be maximally robust, this consortium (or organism) should itself be able to use any of a large set of carbon sources that may be available in the relevant PCE-polluted environments. We shall call this consortium 'the fermenting guild'. We use the word 'guild' because it refers to a set of organisms each of which carries out the well-defined task of providing electrons at sufficiently negative redox potential to sustain the reduction of PCE by *D. hafniense* Y51. The organisms of the guild may differ in what they use as their own electron donor. Organic matter such as molasses, a waste product from the sugar industry with desired characteristics for bioremediation such as uniform distribution throughout the aquifer and their relatively low cost (Henry, 2010), could provide the carbon, Gibbs energy and electron sources to some members of this guild. Fig. 1 uses glucose as exemplar, but the intended robust fermenting guild should use a variety of electron sources alternative to glucose: thereby a guild would make the system more robust than a single organism would. Alternatively, *D. hafniense* Y51 could itself play this role (without fermenting guild) but with addition of suitable electron donors for this strain, but we would guess that this would make the bioremediation network system less robust functionally. Diversity within the electron supply guild should increase the robustness.

The second consortium should further reduce the dichloroethylene (DCE) produced by *D. hafniense* Y51 (in the form of *cis*-DCE), to the harmless ethene. *D. hafniense* strain Y51 does not have the capability of degrading PCE beyond (*cis*)-DCE, but *Dehalococcoides mccartyi* does, and we use it in our model as an example. However the accumulating *cis*-DCE and VC could be also overcome through anaerobic or aerobic oxidative bacterial activities (**Chapter 1**). We will call this the 'downstream bioremediation guild'. As shown in Fig. 1A, the role played by *D. hafniense* could also be played by a third guild, tasked with the reduction of PCE to DCE, i.e. a 'PCE to DCE bioremediation guild', to which *D. hafniense* Y51 could be or could be made to be, the main contributor.

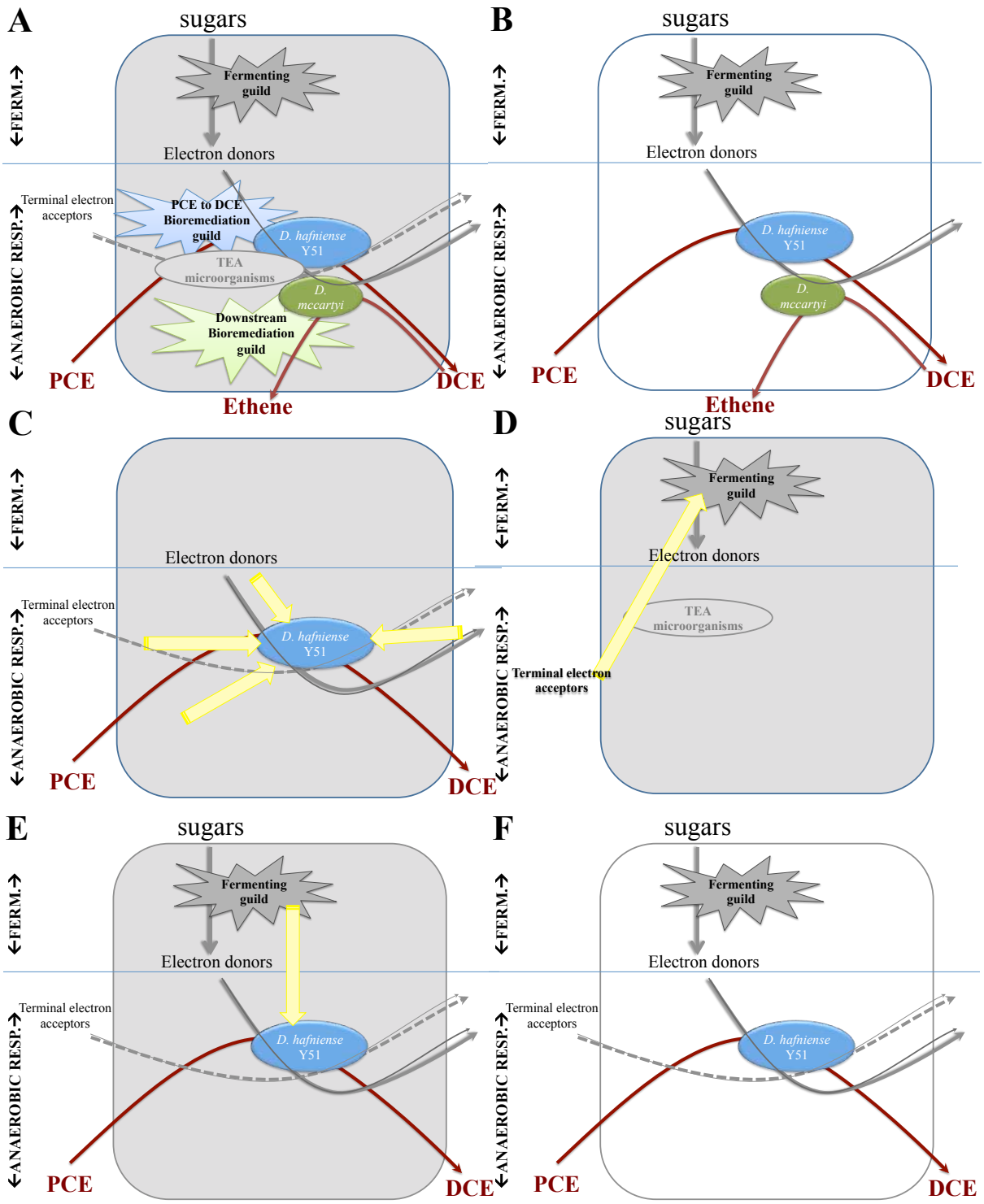
In **Chapter 1** we reviewed that perchloroethylene (PCE) is a common groundwater

contaminant due to its vast industrial production and associated spills (Häggblom and Bossert, 2003). The toxic effects and persistence of PCE and its penultimate degradation products (TCE, (*cis*-)DCE and VC (ethene, the final product, is harmless), has led to extensive efforts to remove these pollutants from contaminated groundwater. Standard remedial methods to clean-up PCE polluted sites are expensive and invasive to the environment, and thereby unsustainable (Wenning et al., 2006; Mccarty, 2010). To date, biodegradation through anaerobic reductive dechlorination (RD) by organohalide respiring bacteria (OHRB), is the most cost-effective and environmentally benign way to remediate PCE pollution (Bradley and Chapelle, 2010; **Chapter 1**).

RD of CEs can occur naturally, i.e. without human intervention. This is called natural attenuation. If this is not fast enough, fermentable organic substrates (glucose in Fig. 1) may be added to stimulate indirectly the growth and activity of indigenous OHRB (this action is called biostimulation). However, when OHRB are absent from a polluted site or insufficiently active to complete RD of PCE, appropriate exogenous OHRB (such an action is called bioaugmentation) may be added (**Chapter 1**). *Desulfitobacterium hafniense* strain Y51 is an efficient PCE-respiring and highly versatile bacterium that can be cultured relatively easy in batch and of which the genome has been sequenced (Suyama et al., 2001; Furukawa et al., 2005; Peng et al., 2012). Furthermore, strain Y51 (unlike other relevant OHRB such as *Dehalococcoides mccartyi* or *Dehalobacter*) is not dependent on halogenated compounds for growth (it is a non-obligate OHRB) and is self-sufficient for the corrinoid cofactor vitamin B_{12} (Nonaka et al., 2006), which is essential for dechlorination (Smidt and de Vos, 2004). We therefore took *D. hafniense* Y51 as the central microorganism in this thesis and in our network design (Fig. 1).

D. hafniense Y51 however, cannot directly use the sugars (e.g. glucose) to be provided, as carbon and energy source. It requires smaller substrates such as lactate, formate, or hydrogen (**Chapter 2**, **Chapter 4**). Our design therefore includes a microbial guild that converts the sugars to these smaller substrates (see above). In order for it not to completely respire the sugars and to be able to survive anaerobically, this consortium should engage in fermentation. The core of our proposal to deal with PCE-polluted sediment or groundwater is indicated in Fig. 1B: The sediment polluted with PCE is to be (i) biostimulated by sugar addition, (ii) further bioaugmented with a consortium (guild) of microorganisms which ferment sugars to smaller products such as lactate, formate and hydrogen (fermenting guild), (iii) bioaugmented with *D. hafniense* Y51, and (iv) bioaugmented further by a DCE to ethene-degrading organism (in this case we have used *Dehalococcoides mccartyi*). We use

the term ‘triple-bioaugmentation’ to refer to the fact that the bioaugmentation essentially consists of three components.



(cont. Fig. 1)

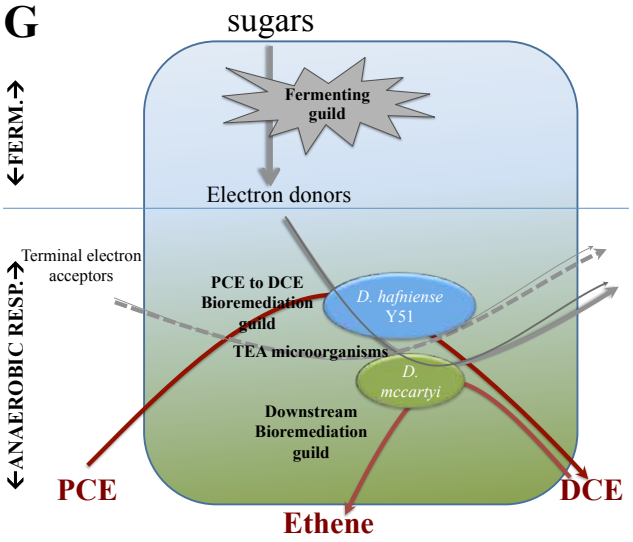


Fig. 1. Proposed microbial ecology design by systems biology approach for efficient reductive dechlorination of PCE using *D. hafniense* Y51 as central component. A) Competing guilds in a possible PCE bioremediation scenario upon triple-bioaugmentation plus biostimulation (sugar addition). B) Proposed "ideal" robust network of triple-bioaugmentation plus biostimulation for removal of PCE to ethene. C) Main interactions of strain Y51 with its immediate environment (PCE, alternative TEAs, electron donors and environmental stresses) that may affect its RD efficiency. D) Indirect effect of terminal electron acceptors (TEAs) on the fermenting guild (identity and fermentation products). E) Influence of fermenting guild on the RD of PCE by *D. hafniense* Y51. F) Trial of dual-bioaugmentation plus biostimulation on PCE-polluted sterile river sediments. G) Trial of dual-bioaugmentation plus biostimulation on PCE-polluted native river sediments. The words "Ferm." and "Anaerobic Resp." indicate fermentation and anaerobic respiration, respectively.

The box in Fig. 1B is white, indicating that there would be nothing else than this. However, our design is meant to operate in a real, polluted site. Sites polluted with PCE already contain microbial ecosystems that may also compete both with *D. hafniense* Y51 and with the fermenting guild, and both for initial substrates (here glucose) and for the electron donors to *D. hafniense* Y51. These native microbiota may engage in many metabolic processes, including fermentation, TEA reduction, homoacetogenesis, and even PCE degradation. We have to take these competing guilds into account in our design. The complete set of guilds is complex, indeed an ecosystem in itself, part of the total ecosystem (Fig. 1A). In the present study we have focused on the dual-bioaugmentation part of the system that reduces PCE to DCE and have less considered the downstream bioremediation guild. Fig. 1 reflects this focus.

Testing the design of the dual bioaugmentation network for PCE removal to DCE

A biological network consists of both components and interactions, and both determine its performance. Components and interactions can often be analyzed in a more controlled way by decomposing the network into smaller sub-networks, where only two components interact

or in which a single component is studied in terms of its interactions. We have here implemented this systems biology approach in our design of microbial ecology (Fig. 1). We studied the interaction of the central component *D. hafniense* Y51 with its immediate environment: *D. hafniense* Y51 inclusive of the PCE, of possible alternative terminal electron acceptors (TEAs), of its electron donors as well as of environmental stresses (the yellow arrows in Fig. 1C). We also examined whether the dominant TEAs were responsive to what happens downstream in the proposed network (the yellow arrow in Fig. 1D), expecting that this interaction should be negligible (but it wasn't, see **Chapter 3**). We were next interested (Fig. 1E) in the influence the fermenting guild might have on *D. hafniense* Y51, which was again substantial (**Chapter 4**). We then examined whether in principle all these interactions together might lead to a robust network for the removal of PCE from the environment and we thought it would: The fermenting guild should supply *D. hafniense* Y51 with suitable electron donors (e.g. formate, lactate) it would need to reduce the PCE. *D. hafniense* Y51 should be able to use these electron donors (for example, if fermenting guild just produces acetate as product from fermentation, strain Y51 would not be able to perform RD). The system should be robust if the fermenting guild was treated under a specific set of redox conditions (see below). In order to examine whether indeed the designed network would work in experimental practice, we put it in place in sediment, in particular in a river sediment, but then in the absence of the native microbiota that may or may not contain natural members of the PCE to DCE bioremediation guild: we first sterilized the sediment (Fig. 1F). In this experimental system, the design worked well; both *D. hafniense* Y51 and the fermenting guild were effective (see **Chapter 5**).

The litmus test of course was the true live test, i.e. with the native microbial communities (which may or may not have remediation activity themselves) live and kicking in the sediment (Fig. 1G). It was here where the design started to stutter: yes, *D. hafniense* Y51 could be shown also to engage here in PCE removal, but this time it was limited to a contribution early on (the onset of RD); as time proceeded the bioremediation by the OHRB already present in the sediment was almost as strong (see **Chapter 5**). The additional bioaugmentation (dual-bioaugmentation) by fermenting guilds trained under denitrifying or iron reducing conditions did not follow the same trend as observed under controlled liquid medium conditions (**Chapter 4**). Reasons why the assay was not as satisfactory as expected will be discussed in the following sections.

We conclude that we have been able to achieve the main aim of this thesis, i.e. understand the environmental factors and interactions (abiotic and biotic) that control the

activity of *Desulfitobacterium hafniense* Y51, design a new strategy for PCE-bioremediation using this OHRB, and test it experimentally. We also achieved a second aim, i.e. validation that this design should be able to work at least in some cases, as we shall discuss further below. We shall however first discuss each of the above test phases individually in the context of the overall design and the scientific literature.

***D. hafniense* Y51 and its interactions**

Natural environments are often characterized by low concentrations of essential nutrients (e.g. carbon, nitrogen and TEAs) for microbial growth, as the result of the limited bioavailability of these resources and the metabolic activities of indigenous microbial populations. In effect the microbes will grow maximally until at least the concentration of one of their essential resources drops to levels not too far above the Monod constant, and growth will then continue at a rate proportional to the influx rate of that nutrient and the growth yield. Consequently, growth of bacteria is slow in most environments (Harder and Dijkhuizen, 1983; Langwaldt et al., 2005; Egli, 2010), as they are subjected to nutrient limitation/starvation and other environmental stresses. Insight into the robustness to stresses that will prevail under these natural conditions was limited for *D. hafniense* Y51, the strain that we considered for bioaugmentation. In **Chapter 2**, *D. hafniense* Y51 was therefore exposed in chemostats to environmentally relevant limiting conditions (in terms of carbon and electron-acceptor limitations) . We though that these conditions might cause this strain to resemble its behavior in natural habitats. The exploration of transcriptomic and proteomics under limiting conditions showed that *D. hafniense* Y51 possesses besides metabolic versatility, an even stronger physiological flexibility **(Chapter 2)**. Strain Y51 applies specific strategies so as to escape unfavourable environments via sporulation related enzymes. Besides and unexpectedly, under free energy or electron donor limitations, *D. hafniense* Y51 appears to optimize its use of available sources of free energy and carbon, i.e. this strain is able to switch between reduction-oxidation reactions with external electron acceptors and fermentation, depending on the conditions applied: “Omic” analyses suggested that strain Y51 might experience relief from carbon catabolite repression of alternative catabolic pathways during electron donor and/or acceptor limitations. This phenomenon of the “relief of carbon catabolite repression” has also been observed for other bacteria that are relevant to pollution degradation such as *Geobacter metallireducens* (Marozava et al., 2014a), enabling fast responses when new carbon sources become available (Egli, 2010; Marozava et al., 2014b). The absence of repression of catabolic pathways might be an efficient strategy under

limiting conditions (Hoehler and Jorgensen, 2013). Our **Chapter 2** herewith showed that strain Y51 has a great potential for survival under environmental conditions of substrate stress and hence for *in situ* bioremediation of PCE.

It is relevant to compare this with other bacteria that might also reduce PCE, such as the *Dehalococcoides mccartyi*, which we actually found to be fairly abundant in the live sediments in which we implemented our design (**Chapter 5**). These species would have the advantage over strain Y51, that they degrade PCE all the way down to the harmless ethene, rather than to the still toxic *cis*-DCE (Löffler et al., 2013). However, *D. mccartyi* is known as fastidious organism since it grows slowly (0.8 to 3 days doubling time; much slower than the doubling time of strain Y51, 9.3 hours, **Chapter 2**), to low densities and it needs specific nutritional requirements (H_2, acetate, vitamin B_{12} and organohalides as TEAs) which may restrict its survival (Löffler et al., 2013; **Chapter 1**). Despite this, *D. mccartyi* often thrives in anaerobic groundwater habitats, driving the RD of large amounts of organohalides worldwide (Atashgahi et al., 2016). Hence in recent years *D. mccartyi* strains have received much attention in relation to their application in the bioremediation of CEs (Taş et al., 2010). The maximum specific substrate utilization rates (q_{max}) and half-saturation constants (K_S) for PCE reported for *D. mccartyi* strains are lower than those of other OHRB-like *Desulfitobacterium* sp. (Becker, 2006; Huang and Becker, 2009). Thus, the higher PCE dechlorination capacity of *Desulfitobacterium* could provide it with an advantage in the competition with *Dehalococcoides* when PCE (and TCE) is present at high concentrations, speeding up the process as compared to a situation with *Dehalococcoides* as sole OHRB present (Becker, 2006; Huang and Becker, 2009). Enabling the coexistence of these OHRB might therefore be an attractive and effective strategy for bioremediation of PCE contaminated sites.

Redox conditions are recognized as one of the major factors that affect the efficiency of anaerobic RD of PCE (Bradley, 2000). Microorganisms tend to use the electron acceptors that enable the largest free energy harvest preferentially. Or, even if they would not, the ones with the highest free energy gain, typically have the highest growth yield and hence for the same substrate supply rate, they will outgrow the ones with lower free energy harvest, exponentially. Consequently, in the presence of oxygen the reduction of CEs is an unfavorable process; organisms engaging in this will be outgrown by organisms using oxygen as electron acceptor, either for the above mentioned reason or because they do not protect themselves against reactive oxygen species such as superoxide anion radical ($\cdot O^-_2$) by taking out the oxygen by a high affinity cytochrome oxidase (Bradley, 2000).

The range of redox potentials for CE reduction (**Chapter 1**, Fig. 2) suggests that RD is

less favorable for microorganisms than denitrification but more so than nitrate to nitrite reduction, nitrate ammonification and iron reduction. As observed in the biodegradation experiments described in **Chapter 4**, the removal of nitrate, ferric iron and sulfate from their respective enrichments by the addition of glucose was required to avoid competition for reducing equivalents between strain Y51 and other TEA microorganisms present in the consortia (nitrate-, iron-, sulfate-reducing and methanogens). This agrees with previous studies (Henry, 2010). Depletion of TEAs was also necessary in order to prevent the preferential use of nitrate over PCE by the strain Y51 (Furukawa et al., 2005). The addition of fermentable substrates such as molasses might help to deplete other electron acceptors that might compete with PCE respiration (Henry, 2010; **Chapter 1**). However, introducing PCE-respiring conditions does not ensure total disappearance of competitors for electron donors as observed in **Chapter 4**. Despite the introduction of PCE and the different hydrogen thresholds for OHRB and homoacetogens (**Chapter 1**, Fig. 3), both co-existed in consortia derived from sulfate-reducing and methanogenic conditions (SO4/Y51 and CO2/Y51, respectively), allowing stable, but incomplete, long-term PCE dechlorination activity as shown during continued propagations **(Chapter 4)**.

Plasticity of the fermenting guild

In our design, the task of the fermenting guild was to ferment the added glucose into electron donors that could be used by *D. hafniense* Y51. This was a concern as (see **Chapter 2**) we had just shown that *D. hafniense* Y51 may be very versatile but not universally so: it could use neither acetate nor propionate as electron source (**Chapter 4**). Acknowledging this, we searched ways in which we could have fermenting guilds with different characteristics, characteristics that might affect their utility as providers of electron donors to *D. hafniense* Y51 and hence, the efficiency in RD. Since the redox products in fermentation are likely to depend on the redox exit pathways present, and since these would be determined differently also in a respiratory growth phase with electron acceptors of different midpoint potentials, we decided to try to 'train' the potential fermenting guild in the presence of different such active electron acceptors, i.e. nitrate, ferric iron, sulfate and carbon dioxide. A corollary to this is that under these different conditions different hydrogen activities (partial pressures) are supposed to be present, again reflecting different effective redox potentials (**Chapter 1**, Fig. 2). Besides this, in subsurface plumes of organic matter such as landfill leachates, frequently changes in environmental redox conditions occur (Christensen et al., 2000). Hence, determining to what extent in a particular soil, the redox conditions affect the local

fermenting guild and the fermentation products is particularly significant for the *in situ* anaerobic bioremediation of CEs.

Redox conditions are known to affect respiring microorganisms (besides OHRB) such as nitrate-, iron-, sulfate-reducers and methanogens (Stams, 1994; Schink, 1997). But unexpectedly because they did not carry out a net external redox reaction, also the fermenting consortia we examined were affected by the reducing conditions they were exposed to, as shown in **Chapter 3**. This finding is relevant to this thesis since generally OHRB rely on the fermenting guild for supply of electron donors and vitamins, OHRB are more robust within this guild (Maymó-Gatell, 1997; Duhamel et al., 2004; Holmes et al., 2006; Löffler et al., 2013), and more importantly, control of the rate of PCE reduction in anaerobic microbial networks, may reside at least partly with the fermenting microorganisms (Röling et al., 2007). In **Chapter 3**, the time-courses of glucose fermentation and electron-acceptor reduction obtained in enrichment cultures under four different redox conditions were correlated with phylogenetic information derived from 16S rRNA gene-based pyrosequencing analysis. This revealed that redox conditions did have a strong influence on the identities of the fermenting species and on their type of fermentation, in contrast to our hypothesis based on thermodynamics (**Chapter 3**, Table 1).

We suggest that the differences in glucose metabolism between the four environmentally relevant redox conditions (nitrate-, iron-, sulfate-reducing and methanogenic) were due to the interaction with terminal electron accepting microorganisms (defined, in turn, by the dominant redox condition) that consume the fermentation products and prevent the product inhibition of further fermentation by their accumulation, although thermodynamics remains exergonic. Indeed, as shown in **Chapter 3**, there are various ways in which glucose can be fermented with different relative concentrations of the fermentation products resulting (e.g. lactate, acetate, formate, hydrogen). Fermenting organisms may be inhibited by hydrogen, acetate and other volatile fatty acids they produce (Fukuzaki et al., 1990a, 1990b; Schink, 1997), requiring collaboration with other anaerobic respiring microorganisms that consume their fermentation products, especially under energetically less favourable redox conditions (Schink, 1997; Stams and Plugge, 2009). Co-habitation with microorganisms that consume the fermentation products largely removes the inhibitory effects of the latter, enabling the fermentative bacteria to shift their fermentation patterns towards higher free energy and growth yields (Iannotti et al., 1973; Scheifinger et al., 1975; Chen and Wolin, 1977). Hence, and as shown in **Chapter 3,** *thermodynamics ruled, but indirectly, through the cohabiting species*. The different fermenting microbial communities

related to specific redox conditions might potentially influence activity of strain Y51 and thus biodegradation, and this will be discussed in the next sections.

***D. hafniense* Y51 responding to the fermenting guild**

The results obtained in **Chapter 3** raised the question if the identity and behavior of the fermenting guild with different redox history would influence strain Y51 in relation to growth, survival and functioning; and thus also with respect to biodegradation of PCE. So, the distinct glucose-fermenting microorganisms enriched under either nitrate-, iron-, sulfate-reducing or methanogenic conditions **(Chapter 3)** were associated to strain Y51 **(Chapter 4)** during several propagations under PCE-respiring conditions. All consortia were capable of supplying *D. hafniense* Y51 with suitable electron donors for its growth and for de-chlorination of PCE to *cis*-DCE. This may be expected based on the variety of electron donors that strain Y51 can use (compared to obligate OHRB) (Suyama et al., 2001; Villemur et al., 2006; Peng et al., 2012), including butyrate and hydrogen (**Chapter 4)**. However, the redox history of the fermentation guilds strongly affected the extent of dechlorination upon augmentation with *D. hafniense* Y51 **(Chapter 4)**. The observed differences in dechlorination capacities related to differences in community composition, which in turn determined the availability of carbon and free energy for strain Y51. This may either have been due to failure of the fermenting organisms to produce sufficient electron donors for strain Y51 or due to the presence of potential competitors for the reducing equivalents (**Chapter 4**).

The introduction of PCE-respiring (i.e. using PCE as electron acceptor) conditions led a substantial shift in the composition of all consortia (decrease in diversity, disappearance of TEA microorganisms) but homoacetogens remained present in consortia derived from the least favourable reducing conditions (**Chapter 4**), as mentioned above. Sulfate-reducing and methanogenic conditions are characterized by relatively high hydrogen concentrations (Lovley, 1988; Luijten et al., 2004), while even higher hydrogen thresholds are required for acetogenic metabolism (Luijten et al., 2004). Hence, these homoacetogens appear to be carried over and maintained during continued propagation of the Y51-amended sulfate-reducing and methanogenic consortia. Indeed we find rather substantial acetate production of these two types of consortia.

Under our experimental conditions applied in **Chapter 4** (liquid defined medium and a specific PCE concentration of 0.8 mM), extra addition of carbon source (i.e. glucose in excess) should provide enough reducing equivalents to complete (at any time) RD of PCE to *cis*-DCE. However under field conditions the issue is trickier. Many reports on managing the

competition between OHRB, homoacetogens and methanogens in favor of RD are available (e.g. Fennell et al., 1997; Yang and McCarty, 1998; Yang and Mccarty, 2000; Ziv-El et al., 2012a, 2012b). However, applying biostimulation in the field is still an engineering challenge where ensuring adequate substrate, loading rates and an effective distribution method (Henry, 2010) is needed to optimize and sustain PCE bioremediation over time. For example, the presence of excess H_2 may lead to substrate competition with homoacetogens and methanogenic Archaea in the consortia that also can use H_2, albeit at a higher substrate threshold than *Desulfitobacterium* (**Chapter 1**, Fig. 3) (Yang and McCarty, 1998). OHRB are more competitive with methanogens when the H_2 concentration are low (Fennell et al., 1997). However, it was also observed that a suppression of competing homoacetogens, such as *Acetobacterium*, which can provide vitamin B_{12} cofactor and acetate as carbon source to *D. mccartyi*, lead to a decreased RD efficiency (Ziv-El et al., 2012b). Therefore, field data collected during pilot testing may always be required for a direct and more definitive indication of the effectiveness of a particular substrate/loading rate, and whether it is appropriate for enhancing RD or whether modifications should be called for.

If left to itself, the designed network works

Reduction of CEs under field conditions occurs in a complex microbial context, where multiple functional groups interact (Macbeth et al., 2004; Miller et al., 2007) and OHRB members constitute just a small percentage of the microbial community (Maphosa et al., 2012). In addition, RD in the environment does not only depend on metabolic capacities of individual OHRB, but also on their interactions with other community members such as fermenting organisms (Röling et al., 2007), which generate, by fermentative activities, electron donors (hydrogen, organic acids like lactate) for OHRB (Kao et al., 2003; Maphosa et al., 2010a). But up to date little attention has been paid to this functional group (Daprato et al., 2007) and to their effect on PCE degradation, despite that in the natural environment the growth and efficiency of OHRB (in this case strain Y51) depend on the activity of the non-dechlorinating bacterial guild **(Chapter 1,** Fig. 3**)**.

We observed in **Chapter 4** that *D. hafniense* Y51 was more robust, achieved higher densities, and dechlorinated more rapidly when growing in a defined liquid culture within appropriate fermenting consortia (i.e. absence of competitors and suitable electron donors) such as NO3_c/Y51 and FeIII_a/Y51, than as monoculture. This may be related to the growth factors (e.g. nucleotides, amino acids) and/or vitamin B12 corrinoid that members of the community provide to strain Y51, avoiding the need of synthesizing these compounds *de*

novo. If such is the case, the rate of glucose-stimulated de-chlorination should be enhanced by additional bioaugmentation with co-cultures of strain Y51 and with appropriate fermenting consortia.

Addressing this subject, in **Chapter 5** we then examined the interaction of strain Y51 and the fermenting consortia in artificially PCE-polluted river sediments. Eliminating the complexity of the endogenous microbial community in the sediment by autoclaving the latter **(Chapter 5),** the bioaugmented Y51-containing consortia showed comparable results to the ones obtained in the defined basal medium (degradation rates, fermentation products and community composition) **(Chapter 4)**, suggesting a predictable behavior of bioaugmented communities in an *in situ* bioremediation scenario. However, and unexpectedly, strain Y51 did not depend on other microorganisms for their supply of electron donors and growth factors and corrinoids in autoclaved sediments **(Chapter 5)** unlike the results obtained in defined basal medium **(Chapter 4)**. The thermal treatment applied during autoclaving may have released soluble organic carbon, hence *D. hafniense* Y51 could grow without "help" from other organisms, using the released compounds and coupling this to RD.

The autochthonous ecosystem may interfere

The sediment microcosms studies done in the **Chapter 5** with the autochthonous ecosystem present did not indicate the same trend as observed in autoclaved sediments. Although the exact cause has not been confirmed, the high functional and physiological diversity of the sediments may have been a key factor. Previous studies (van Elsas et al., 2011) have demonstrated a negative correlation between the diversity of the soil microbiota and survival of the invader. The high diversity of the sediments may have decreased colonization by Y51-containing consortia. This suggest that for more effective, quicker and especially more robust (i.e. reproducible, predictable) bioaugmentation, more work is required on (i) establishing the potential sources for free energy, carbon, nitrogen and electrons, (ii) determining the diversity (and density) of the autochthonous microbial population in terms of fermentation guild, downstream DCE remediation guild and competing guilds, and then (iii) establishing the appropriate inoculum size (number of cells) of the Y51-containing consortium used for bioaugmentation. The importance of inoculum size during bioaugmentation with *D. mccartyi* has already been reported. Delgado et al. (Delgado et al., 2014) observed that when *D. mccartyi* was present in low numbers in bioaugmented microcosms, it was not competitive enough for electron donors with methanogens and other hydrogenotrophs present, showing *cis*-DCE stall. However, after a round of enrichment where *D. mccartyi* achieved high

numbers, the organism (or its guild) was able to compete for reducing equivalents for RD, with subsequent (and desirable) ethene production.

Other reasons for the inability of augmented microorganisms to perform their desired activities or their survival, both in natural environment as in "live" microcosms, could be numerous. These may include predation, inadequate environmental conditions to which they are not adapted or inefficiency to compete for substrates (Goldstein et al., 1985). In this sense, based on the phylogenetic information of **Chapter 5** we did not find potentially predating species and, environmental (i.e. microcosm) conditions seemed adequate when compared to data obtained in bioaugmented sterile sediment cultures. In fact, the inability of strain Y51 to compete for substrates was discarded, since despite a low capacity of colonization of Y51-containing consortium (based on rRNA) due to the high diversity of the soil (all ecological niches were already occupied), this strain was still identified as the only OHRB responsible of PCE to *cis*-DCE degradation **(Chapter 5)**.

A paradoxical limitation to bioremediation of PCE is pampering, i.e. the provision of too much of substrates that are much liked by the bioremediation guild. However, we observed in **Chapter 2** that *D. hafniense* Y51, induces alternatives when confronted with limitations: fermentation of the excess lactate and disproportionation of the excess fumarate as a response to electron acceptor and electron donor limitation, respectively; performing a maximum use of available sources of carbon and energy. Hence, it may be useful if strain Y51 grows under carbon or energy limitation prior to its introduction into PCE polluted sites, this 'pre-adaptation' may prepare to this bacterium for future environmental constraints.

Lesson to learn: precision bioremediation

In **Chapter 5** we found that our design for PCE removal from (artificially polluted) sediment by the application of the fermenting consortium, did work well when the endogenous microorganisms were not allowed to play ball, i.e. after sterilization. When the endogenous microbiota was allowed to be active, the bioaugmentation with the fermenting guild worked inefficiently with respect to the PCE removal rate. It may seem that this showed that in actual practice the fermenting part of our design will not work, but we doubt that this is the case.

We think that these experiments show that the effectiveness of the design will differ between pollution sites. One should keep in mind the two microbiology laws of Beyerinck recognized by Baas-Becking (Quispel, 1998), i.e. 'everything is everywhere' and 'the environment selects'. Indeed, in any soil, one should expect PCE bioremediation potential to be present,

where the activity of the corresponding guild depends on pre-exposure. Sites that have been pre-exposed for a long time to PCE may already have developed substantial PCE removal activity inclusive of a fermenting guild supporting that activity. Indeed, biogeographic studies have confirmed the presence of OHRB such as *Desulfitobacterium* or *Dehalococcoides* in the majority of samples originated from sites contaminated with CEs compounds. Naturally occurring *Dehalococcoides* spp. and *Desulfitobacterium* spp. frequently coexist in CE polluted sites (Maphosa et al., 2010a; Rouzeau-Szynalski et al., 2011). This is understandable from the observation that these guilds are also found in pristine environments (Hendrickson et al., 2002; Rouzeau-Szynalski et al., 2011; Atashgahi et al., 2016), confirming the laws of Baas-Becking and Beyerinck (Quispel, 1998). Little information is available about associated fermenting guilds (Daprato et al., 2007), but their presence should again be suspected.

In our study (**Chapter 5**) we should perhaps first have determined what level of bioaugmentation was required to be able to offset the biostimulation of OHRB and their RD activity in any indigenous microbial population for the Appels site. We had not found evidence of CE pollution in sediments from the Scheldt site in Appels, but this is always an uncertain piece of evidence: CEs may be absent either because there has never been a spill, or because there has been one and the PCE removal guild has been removing it and has thereby become amplified. This estuary has been subject to intense anthropogenic disturbance, receiving elevated amounts of organic carbon per year (Hyacinthe and Van Cappellen, 2004). Hence, our implicit assumption that there had never been a PCE spill may well have been naïve. Our cultivation independent analysis of the sediment did find evidence of native *Dehalococcoides* and *Desulfitobacterium*, although the latter did not display RD activity (**Chapter 5**). In such sites with intrinsic RD, as occurred in our sediments, bioaugmentation with *D. hafniense* Y51 may not be essential, but it may be useful in some cases to reduce cost or duration. Often, natural acclimatization by the native microbial population may necessitate a longer time due to a higher lag-period for onset of dechlorination, leading to prolonged bioremediation (Lendvay et al., 2003). Indeed, in non-bioaugmented microcosms we observed a lag time before RD occurred as compared to Y51-augmented sediments (**Chapter 5**). The duration of the experiment may have been too short to really compare degradation rates upon bioaugmentation with those upon biostimulation only.

At sites without a PCE contamination history, i.e. in the case of acute PCE pollution of a pristine environment, endogenous PCE dechlorinating microorganisms may be rare as may be suitable fermenting consortia (‘suitable’ concerning to providing adequate reducing

equivalents for RD). During a spill, PCE migrates into the subsurface as a dense non-aqueous phase liquid, producing a highly contaminated zone that serves as a long-term source of PCE dissolved in water (Christ et al., 2004). This toxicity may diminish the native microbial diversity: PCE concentrations in excess of 0.1 mM inhibit some microbial groups. These include methanogens and homoacetogens (Yang and Mccarty, 2000) as well as other TEA microorganisms and fermenting organisms, although the latter two guilds (especially fermenting organisms) tend to be more tolerant to PCE toxicity (Bowman et al., 2009; Koenig et al., 2014). However, *D. hafniense* Y51 is capable of dechlorinating PCE at concentrations as high as 1 mM (i.e. close to water saturation) and the fermenting guilds used in our study were capable of functioning in the presence of PCE. In this case as well as at sites where thermal treatment had been applied (in order to mobilize dense non-aqueous phase liquids and to reduce competition with indigenous microorganisms) (Friis et al., 2006; Fletcher et al., 2011), our design of dual-bioaugmentation should be effective through both legs of its bioremediation strategy.

The lesson to learn is that one should determine for each polluted site what the level of PCE contamination is and what micro-ecosystem is already present. With the molecular methodology used in our studies this should be possible and is becoming affordable even at the microeconomic level. Next one should integrate the data of (i) the preexisting site, (ii) the level of pollution, (iii) the properties of the fermenting guild to be used for bioaugmentation, (iv) the properties of the bioremediation guild to be used for bioaugmentation, (v) the properties of various competing guilds, and (vi) the possibility of a 'pre-adaptation' under limiting conditions. Because of complex time and space dependencies such data integration would require an ecological systems biology much of which still has to be developed. Because any two polluted sites are likely to differ in at least one but probably in many of these aspects, the data integration will need to be done slightly differently for different sites. Indeed, the lesson is similar to that of individualized or precision medicine (Westerhoff et al., 2015) and hence we would call such future approach 'precision bioremediation'.

Precision bioremediation workflow: a start

Our study may serve to lead the way into precision bioremediation. In short, when a site is reported to be polluted, one may start up the following procedure (modified from (Stroo et al., 2010)):

- Assessing the RD activity of the indigenous microbial population and the rate at which

this would be activated by the provision of PCE.

- Evaluating and/or modifying environmental conditions (through biostimulation) to ensure that the proper geochemical conditions are achieved to enhance RD.
- Confirming that bacteria used for the bioaugmentation possess the ability to compete with the native microbial communities and to degrade the target pollutant completely.
- Ensuring that bacteria used for bioaugmentation will be well distributed over the site (sediment/groundwater), taking into account the effect of predation, adsorption, filtration and nutrient competition.
- Monitoring from replicated field trials and quality controls the treated area, in order to provide a direct indication of the effectiveness of the approach and the possibility to motivate modifications.
- Evaluating in terms of cost and performance.

Ideally, the above information would then be inserted into a mathematical model for bioremediation, which would thereby be made specific for the pollution site that is being considered for bioremediation. That model should then provide advice of an optimal bioremediation strategy and enable 'what if' calculations. The latter should aid the eco-engineer when considering alternatives suggested by human intelligence and experience. And, when inserting additional data deriving from the monitoring of the site as the bioremediation process is in action, the model should adjust its predictions and come with advice for adjustments of the interventions with the site.

Conclusions and future perspectives

The investigation of the (eco)physiology of *D. hafniense* Y51 in this thesis showed that this strain exhibits an extremely flexible metabolism, as well as considerable survival ability and active dechlorination activity. It does this under 'controlled' laboratory conditions as well as under closer to environmental conditions (such as sediment with native microbial communities). This confirms the strain's potential for bioaugmentation strategies.

However, considering bioaugmentation, the composition of the *in situ* microbial community needs to be evaluated in terms of, both the type of fermentation products that can be expected from the fermenting microorganisms and the presence/diversity of microorganisms that compete for these substrates with strain Y51. Above we have presented a workflow for precision bioremediation that takes this into account. With this we expect that the current molecular tool box to monitor CE-contaminated field sites undergoing bioremediation (Maphosa et al., 2010b) in combination with systems microbiology, which

allows to build a comprehensive picture of how microbial networks (both inside and between cells) is functioning (Röling et al., 2010; Röling and van Bodegom, 2014; Röling, 2015), will contribute to improved control over CE degradation through rational engineering and monitoring of microbial community performance.

Collaboration of strain Y51 (PCE to *cis*-DCE specialist) with an appropriate fermenting guild such as the one thriving under iron-reducing conditions, together with *Dehalococcoides mccartyi* (*cis*-DCE to ethene specialist) or the corresponding guild of DCE bioremediation, may be the better approach to clean up PCE polluted sites where autochthonous dechlorinating bacteria are not fast/active enough. But, such bioremediation should be done with the care that scientific progress is now beginning to enable: precision bioremediation.

What remains to be discovered; recommendations for further work

The work in this thesis has been gratifying as it leads to well-defined options and action plans. However, there are also some open ends that deserve further experimental scrutiny. First it should be verified experimentally what we have suggested upon cultivation of *D. hafniense* Y51 under limiting conditions in **Chapter 2**: fumarate disproportionation and lactate fermentation. In addition, the complete upregulation of the Wood-Ljungdahl pathway suggests that autotrophic growth might be possible under strong carbon limitation, although it has not yet been shown that *D. hafniense* Y51 can grow autotrophically (Nonaka et al., 2006). Verification of autotrophic growth by this strain (i.e. growth using CO_2 as carbon substrate) is required in future work.

We have performed the chemostat cultures with fumarate as electron acceptor; this molecule has a similar structure to PCE and TCE but easier to work with (e.g. better solubility, not volatile, not toxic). However, we have to take in account that the pce genes of *D. hafniense* are localized on a transposon and, a gradual loss of these genes might occur in the absence of PCE, as shown in *D. hafniense* TCE1 (Duret et al., 2012). This implies that Y51 bioremediation-cells should be maintained in the presence of CEs in order to keep RD activity.

Although we have explored the interaction of strain Y51 with fermentative consortia in batch cultures (**Chapter 4**), it should be useful to set up continuous culturing with fermenting communities and the OHRB, strain Y51 and *D. mccartyi*, under PCE exposure as close as possible to *in situ* situations. Hydrogen and acetate derived from fermentation, and additional acetate produced by strain Y51 would be the energy source required by *D. mccartyi* to

complete the *cis*-DCE degradation to ethene. Further ecological control analysis (Röling et al., 2007) inferring how changes in fluxes through each of the functional groups of microorganisms in a natural PCE degradation network take place, may then allow the 'manipulation' of such networks and promote biodegradation of PCE in *in situ* applications.

Sediments with high functional diversity decreased the colonization by strain Y51 (**Chapter 5**). Simple experiments as described in (van Elsas et al., 2011) with various soil diversities (that may be achieved by a dilution to extinction approach) or also with various inoculum sizes would help determine the optimal range of cells needed for successful invasion of an existing ecosystem with the bioaugmenting organisms.

Furthermore, additional bioaugmentation studies in a more realistic polluted environment will be required. Indeed, we have used sediments artificially polluted with PCE dissolved in hexadecane in order to perform accurate time-course PCE measurements (**Chapter 5**). Although, it has been demonstrated that hexadecane does not have major effect (at proteomic level) on *Desulfitobacterium hafniense* (strain TCE1) (Prat et al., 2011); the use of a hexadecane phase that allows to keep the aqueous PCE concentration low in cultures may have diminished the PCE toxicity to other microorganisms present (Holliger et al., 1992), but also may have served as carbon source or even have produced toxic effects in some bacteria (Brennan and Sanford, 2002); thereby detracting from reality. Microcosms or lab-scale column experiments using real PCE polluted soil and, finally field pilots should be performed to provide pre-design data on substrate loading requirements, injection well spacing and frequency. This should help evaluate the feasibility of our proposed systems/precision bioremediation.

Concluding remarks

In this study we have tried to look systematically at ways to detoxify PCE polluted sediment-groundwater by using biostimulation plus dual bioaugmentation. We recognized that bioremediation of PCE pollution is a network process and have therefore implemented a new form of systems biology. This was systems biology in that it dealt with networks in biology, but it was more than traditional systems biology in that it transcended the intracellular networks that are mostly the subject of systems biology analyses. An exception has been the ecological control analysis (Röling et al., 2007), but this is still at early stages. The developments discussed here may open up another fertile branch of systems microbial ecology.

In addition to this we may have opened new avenues towards more robust and systematic bioremediation. This will come with new and even more exciting scientific discoveries in this field, and may lead to a cleaner world.